AMENDMENTS TO THE SPECIFICATION

The Examiner has requested that the specification be amended to disclose the material incorporated by reference from the priority documents. The following amendments to the specification are in response to this request, and a Statement Under 37 C.F.R. § 1.57(f), certifying that the amendments contain no new matter, is submitted herewith. Applicants note that references to particular sequence and figure numbers from the specifications of the priority documents have been amended to correspond to those in the instant application. These changes, as well as other minor corrections, have been noted accordingly, by underlining insertions and striking out deletions over the priority applications. Also submitted herewith is an updated Sequence Listing, to which Applicants have appended the sequences in the material incorporated below, and an accompanying Statement Under 37 C.F.R. § § 1.825(a)-(b).

Material Incorporated from U.S.S.N. 08/109,393

Please insert the following at page 5, line 30. This material corresponds to page 3, line 27 to page 4, line 17 of U.S.S.N. 08/109,393.

The invention also features nucleic acids which encode a peptide having B7-2 activity and at least about 50%, more preferably at least about 60% and most preferably at least about 70% homologous with an amino acid sequence shown in ~~Figure 8 (~~SEQ ID NO:2~~)~~ or an amino acid sequence shown in ~~Figure 14 (~~SEQ ID NO:4~~23)~~. Nucleic acids which encode peptides having B7-2 activity and at least about 80%, more preferably at least about 90%, more preferably at least about 95% and most preferably at least about 98% or at least about 99% homologous with an amino acid sequence shown in ~~Figure 8 (~~SEQ ID NO:2~~)~~ or an amino acid sequence shown in ~~Figure 14 (~~SEQ ID NO:4~~23)~~ are also within the scope of the invention. In another embodiment, the peptide having B7-2 activity is encoded by a nucleic acid which hybridizes under high or low stringency conditions to a nucleic acid which encodes a peptide having an amino acid sequence of ~~Figure 8 (~~SEQ ID NO:2~~)~~ or a peptide having an amino acid sequence shown in ~~Figure 14 (~~SEQ ID NO:4~~23)~~.

The invention further pertains to an isolated nucleic acid comprising a nucleotide sequence encoding a peptide having B7-2 activity and having a length of at least 20 amino acid residues. Peptides having B7-2 activity and consisting of at least 40 amino acid residues in length, at least 60 amino acid residues in length, at least 80 amino acid residues in length, at least 100 amino acid residues in length or at least 200 or more amino acid residues in length are also within the scope of this invention. Particularly preferred nucleic acids encode a peptide having B7-2 activity, a length of at least 20 amino acid residues or more and at least 50% or greater homology (preferably at least 70%) with a sequence shown in ~~Figure 8 (~~SEQ ID NO:2~~)~~.

Material Incorporated from U.S.S.N. 08/280,757, Now U.S. Patent No. 6,130,316

Please insert the following after the material incorporated from U.S.S.N. 08/109,393 (above). This material corresponds to column 22, lines 23-36 of U.S.S.N. 08/280,757, now U.S. Patent No. 6,130,316.

In another embodiment, mutations can be introduced into a DNA by any one of a number of methods, including those for producing simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases, to generate variants or modified equivalents of B lymphocyte antigen DNA. For example, changes in the human B7-2 cDNA sequence shown in ~~FIG. 8 (~~SEQ ID NO:l~~)~~ or murine B7-2 cDNA sequence shown in ~~FIG. 14 (~~SEQ ID NO:3~~22)~~ such as amino acid substitutions or deletions are preferably obtained by site-directed mutagenesis. Site directed mutagenesis systems are well known in the art. Protocols and reagents can be obtained commercially from Amersham International PLC, Amersham, U.K.

Please insert the following at page 32, line 12. This material corresponds to Example 6 (columns 51-54) of U.S.S.N. 08/280,757, now U.S. Patent No. 6,130,316.

EXAMPLE 6

Cloning and Sequencing of the Murine B7-2 Antigen

A. Construction of cDNA Library

A cDNA library was constructed in the pCDM8 vector (Seed, Nature, 329:840 (1987)) using poly $(A)^+$ RNA from dibutryl cyclic AMP (cAMP) activated M12 cells (a murine B cell tumor line) as described (Aruffo et al., Proc. Natl. Acad. Sci. USA, 84:3365 (1987)).

M12 cells were cultured at 1×10^6 cells/ml in complete culture media, {RPMI 1640 with 10% heat inactivated fetal calf serum (FCS), 2 mM glutamine, 1~~l~~ mM sodium pyruvate, penicillin (100 units/ml), streptomycin sulfate (100 μg/ml) and gentamycin sulfate (5 μg/ml)}, in tissue culture flasks and were activated by 300 μg/ml dibutryl cAMP (Nabavi, N., et al. (1992) Nature 360, 266-268). Activated M12 cells were harvested after 0, 6, 12, 18, 24 and 30 hours.

RNA was prepared by homogenizing activated M12 cells in a solution of 4M guanidine thiocyanate, 0.5% SARKOSYL~~sarkosyl~~, 25 mM EDTA, pH 7.5, 0.13% Sigma anti-foam A, and 0.7% mercaptoethanol. RNA was purified from the homogenate by centrifugation for 24 hour at 32,000 rpm through a solution of 5.7M CsCl, 10 mM EDTA, 25 mM Na acetate, pH 7. The pellet of RNA was dissolved in 5% sarkosyl, 1 mM EDTA, 10 mM Tris, pH 7.5 and extracted with two volumes of 50% phenol, 49% chloroform, 1% isoamyl alcohol. RNA was ethanol precipitated twice. Poly $(A)^+$ RNA used in cDNA library construction was purified by two cycles of oligo (dT)-cellulose selection

Complementary DNA was synthesized from 5.5 μg of dibutryl cAMP activated murine M12 cell poly$(A)^+$ RNA in a reaction containing 50 mM Tris, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 μM dATP, dCTP, dGTP, dTTP, 50 μg/ml oligo$(dT)_{12\text{-}18}$, 180 units/ml RNasin, and 10,000 units/ml Moloney-MLV reverse transcriptase in a total volume of 55 μl at 37° C. for 1 hr. Following reverse transcription, the cDNA was converted to double-

stranded DNA by adjusting the solution to 25 mM Tris, pH 8.3, 100 mM KCl, 5 mM $MgCl_2$, 250 μM each DATP, dCTP, dGTP, dTTP, 5 mM dithiothreitol, 250 units/ml DNA polymerase I, 8.5 units/ml ribonuclease H and incubating at 16° C. for 2 hr. EDTA was added to 18 mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate and with 4 micrograms of linear polyacrylamide as carrier. Following reverse transcription, the reverse transcriptase was inactivated by heating at 70° C. for 10 min. The cDNA was converted to double-stranded DNA by adding 320 μl H_2O and 80 . μl of a solution of 0.1M Tris, pH 7.5, 25 mM $MgCl_2$, 0.5M KCl, 250 μg/ml bovine serum albumin, and 50 mM dithiothreitol, and adjusting the solution to 200 μM each dATP, dCTP, dGTP, dTTP, 50 units/ml DNA polymerase I, 8 units/ml ribonuclease H and incubating at 16° C. for 2 hours. EDTA was added to 18 mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate and with 4 micrograms of linear polyacrylamide as carrier.

2 μg of non-selfcomplementary BstXI adaptors were added to the DNA as follows: The double-stranded cDNA from 5.5 μg of poly(A)$^+$ RNA was incubated with 3.6 μg of a kinased oligonucleotide of the sequence CTTTAGAGCACA (SEQ ID NO:9~~15~~) and 2.4 μg of a kinased oligonucleotide of the sequence CTCTAAAG (SEQ ID NO:10~~6~~) in a solution containing 6 mM Tris, pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 350 μg/ml bovine serum albumin, 7 mM mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine, and 600 units T4 DNA ligase in a total volume of 0.45 ml at 15° for 16 hours. EDTA was added to 34 mM and the solution was extracted with an equal volume of 50% phenol, 49% chloroform, 1% isoamyl alcohol. DNA was precipitated with two volumes of ethanol in the presence of 2.5M ammonium acetate.

DNA larger than 600 bp was selected as follows: The adaptored DNA was redissolved in 10 mM Tris, pH 8, 1 mM EDTA, 600 mM NaCl, 0.1% SARKOSYL~~sarkosyl~~ and chromatographed on a ~~Sepharose~~SEPHAROSE CL-4B column in the same buffer. DNA in the void volume of the column (containing DNA greater than 600 bp) was pooled and ethanol precipitated.

The pCDM8 vector was prepared for cDNA cloning by digestion with BstXI and purification on an agarose gel. Adaptored DNA from 5.5 μg of poly(A)$^+$ RNA was ligated to 2.25 μg of BstXI cut pCDM8 in a solution containing 6 mM Tris, pH 7.5, 6 mM $MgCl_2$, 5 mM NaCl, 350 μg/ml bovine serum albumin, 7 mM mercaptoethanol, 0.1 mM ATP, 2 mM dithiothreitol, 1 mM spermidine, and 600 units T4 DNA ligase in a total volume of 1 .5 ml at 15° for 24 hr. The ligation reaction mixture was transformed into competent E. coli MC1061/P3 and a total of 200×10^6 independent cDNA clones were obtained.

Plasmid DNA was prepared from a 500 ml culture of the original transformation of the cDNA library. Plasmid DNA was purified by the alkaline lysis procedure followed by twice banding in CsCl equilibrium gradients (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. (1987)).

B. Cloning Procedure

In the first round of screening, thirty 100 mm dishes of 50% confluent COS cells were transfected with 0.05 μg/ml activated M12 murine B cell library DNA using the DEAE-Dextran method (Seed et al., Proc. Natl. Acad. Sci. USA, 84:3365 (1987)). The cells were trypsinized and re-plated after 24 hours. After 47 hours, the cells were detached by incubation in PBS/0.5 mM EDTA, pH 7.4/0.02% Na azide at 37° C. for 30 min. The detached cells were treated with 10

μg/ml/human CTLA4Ig and murine CD28Ig for 45 minutes at 4° C.[[.]] Cells were washed and distributed into panning dishes coated with affinity-purified Goat anti-human IgG antibody and allowed to attach at room temperature. After 3 hours, the plates were gently washed twice with PBS/0.5 mM EDTA, pH 7.4/0.02% Na azide, 5% FCS and once with 0. 15M NaCl, 0.01 M Hepes, pH 7.4, 5% FCS. Episomal DNA was recovered from the panned cells and transformed into E. coli DH10B/P3. The plasmid DNA was re-introduced into COS cells via spheroplast fusion as described (Seed et al., Proc. Natl. Acad. Sci. USA, 84:3365 (1987)) and the cycle of expression and panning was repeated twice. In the second and third rounds of selection, after 47 hours, the detached COS cells were first incubated with α-murine B7-1 mAb (16-10A1, 10 μg/ml), and COS cells expressing B7-1 were removed by α-mouse IgG and IgM coated magnetic beads. COS cells were then treated with 10 μg/ml of human CTLA4Ig and murine CD28Ig and murine B7-2 expressing COS cells were selected by panning on dishes coated with goat anti-human IgG antibody. After the third round, plasmid DNA was prepared from individual colonies and transfected into COS cells by the DEAE-Dextran method. Expression of B7-2 on transfected COS cells was analyzed by indirect immunofluorescence with CTLA4Ig.

After the final round of selection, plasmid DNA was prepared from individual colonies. A total of 6 of 8 candidate clones contained a cDNA insert of approximately 1.2 kb. Plasmid DNA from these eight clones was transfected into COS cells. All six clones with the 1.2 Kb cDNA insert were strongly positive for B7-2 expression by indirect immunofluorescence using CTLA4Ig and flow cytometric analysis.

C. Sequencing

The B7-2 cDNA insert in clone 4 was sequenced in the pCDM8 expression vector employing the following strategy. Initial sequencing was performed using sequencing primers T7, CDM8R (Invitrogen) homologous to pCDM8 vector sequences adjacent to the cloned B7-2 cDNA (see Table ~~4II~~). Sequencing was performed using dye terminator chemistry and an ABI automated DNA sequencer. (ABI, Foster City, Calif.). DNA sequence obtained using these primers was used to design additional sequencing primers (see Table ~~4II~~). This cycle of sequencing and selection of additional primers was continued until the murine B7-2 cDNA was completely sequenced on both strands.

TABLE ~~4II~~

T7(F) (SEQ ID NO:11)	5'd[TAATACGACTCACTATAGGG]3'
CDM8(R) (SEQ ID NO:12)	5'd[TAAGGTTCCTTCACAAAG]3'
MBX4-1F (SEQ ID NO:13)	5'd[ACATAAGCCTGAGTGAGCTGG]3'
MBX4-2R (SEQ ID NO:14)	5'd[ATGATGAGCAGCATCACAAGG]3'
MBX4-14 (SEQ ID NO:15)	5'd[TGGTCGAGTGAGTCCGAATAC]3'

MBX4-2F 5'd[GACGAGTAGTAACATACAGTG]3'
(SEQ ID NO:16)

A murine B7-2 clone (mB7-2, clone 4) was obtained containing an insert of 1,163 base pairs with a single long open reading frame of 927 nucleotides and approximately 126 nucleotides of 3' noncoding sequences (~~FIG. 14,~~ SEQ ID NO:17~~22~~). The predicted amino acid sequence encoded by the open reading frame of the protein is shown in SEQ ID NO:4~~below the nucleotide sequence in FIG. 14~~. The encoded murine B7-2 protein, is predicted to be 309 amino acid residues in length (SEQ ID NO:4~~23~~). This protein sequence exhibits many features common to other type I Ig superfamily membrane proteins. Protein translation is predicted to begin at the methionine codon (ATG, nucleotides 111 to 113 of SEQ ID NO:17) based on the DNA homology in this region with the consensus eucaryotic translation initiation site (see Kozak, M. (1987) Nucl. Acids Res. 15:8125-8148). The amino terminus of the murine B7-2 protein (amino acids 1 to 23) has the characteristics of a secretory signal peptide with a predicted cleavage between the alanine at position 23 and the valine at position 24 (von Heijne (1987) Nucl. Acids Res. 14:4683). Processing at this site would result in a murine B7-2 membrane bound protein of 286 amino acids having an unmodified molecular weight of approximately 32 kDa. This protein would consist of an approximate extracellular Ig superfamily V and C like domains of from about amino acid residue 24 to 246, a hydrophobic transmembrane domain of from about amino acid residue 247 to 265, and a long cytoplasmic domain of from about amino acid residue 266 to 309. The homologies to the Ig superfamily are due to the two contiguous Ig-like domains in the extracellular region bound by the cysteines at positions 40 to 110 and 157 to 216. The extracellular domain also contains nine potential N-linked glycosylation sites and, like murine B7-1, is probably glycosylated. Glycosylation of the murine B7-2 protein may increase the molecular weight to about 50-70 kDa. The cytoplasmic domain of murine B7-2 contains a common region which has a cysteine followed by positively charged amino acids which presumably functions as signaling or regulatory domain within an APC. Comparison of both the nucleotide and amino acid sequences of murine B7-2 with the GenBank and EMBL databases yielded significant homology (about 26% amino acid sequence identity) with human and murine B7-1. Murine B7-2 exhibits about 50% identity and 67% similarity with its human homologue, hB7-2. E. coli (DH106/p3) transfected with a vector (plasmid pmBx4) containing a cDNA insert encoding murine B7-2 (clone 4) was deposited with the American Type Culture Collection (ATCC) on Aug. 18, 1993 as Accession No. 69388.

D. Costimulation

$CD4^+$ murine T cells were purified by first depleting red blood cells by treatment with Tris-NH_4Cl. T cells were enriched by passage over a nylon wool column. $CD4^+$ T cells were purified by two-fold treatment with a mixture of anti-MHC class II and anti-CD28 mAbs and rabbit complement. Murine B7-1 (obtained from Dr. Gordon Freeman, Dana-Farber Cancer Institute, Boston, Mass.; see also, Freeman, G. J. et al (1991) J. Exp. Med. 174, 625-631) murine B7-2, and vector transfected COS cells were harvested 72 hours after transfection, incubated with 25 μg/ml mitomycin-C for one hour, and then extensively washed. 10^5 murine $CD4^+$ T cells were incubated with 1 ng/ml of phorbol myristic acid (PMA) and 2×10^4 COS transfectants (Table 5~~III~~). T cell proliferation was measured by 3H-thymidine (1 μCi) incorporated for the last 12 hours of a 72 hour incubation.

TABLE 5~~II~~

	3H-Thymidine Incorporation (cpm)
$CD4^+$ T cells	175
$CD4^+$ T cells + 1 ng/ml PMA	49
$CD4^+$ T cells + COS-vector	1750
$CD4^+$ T cells + COS-B7-1	4400
$CD4^+$ T cells + COS-B7-2	2236
$CD4^+$ T cells + 1 ng/ml PMA + COS-vector	2354
$CD4^+$ T cells + 1 ng/ml PMA + COS-B7-1	67935
$CD4^+$ T cells + 1 ng/ml PMA + COS-B7-2	43847